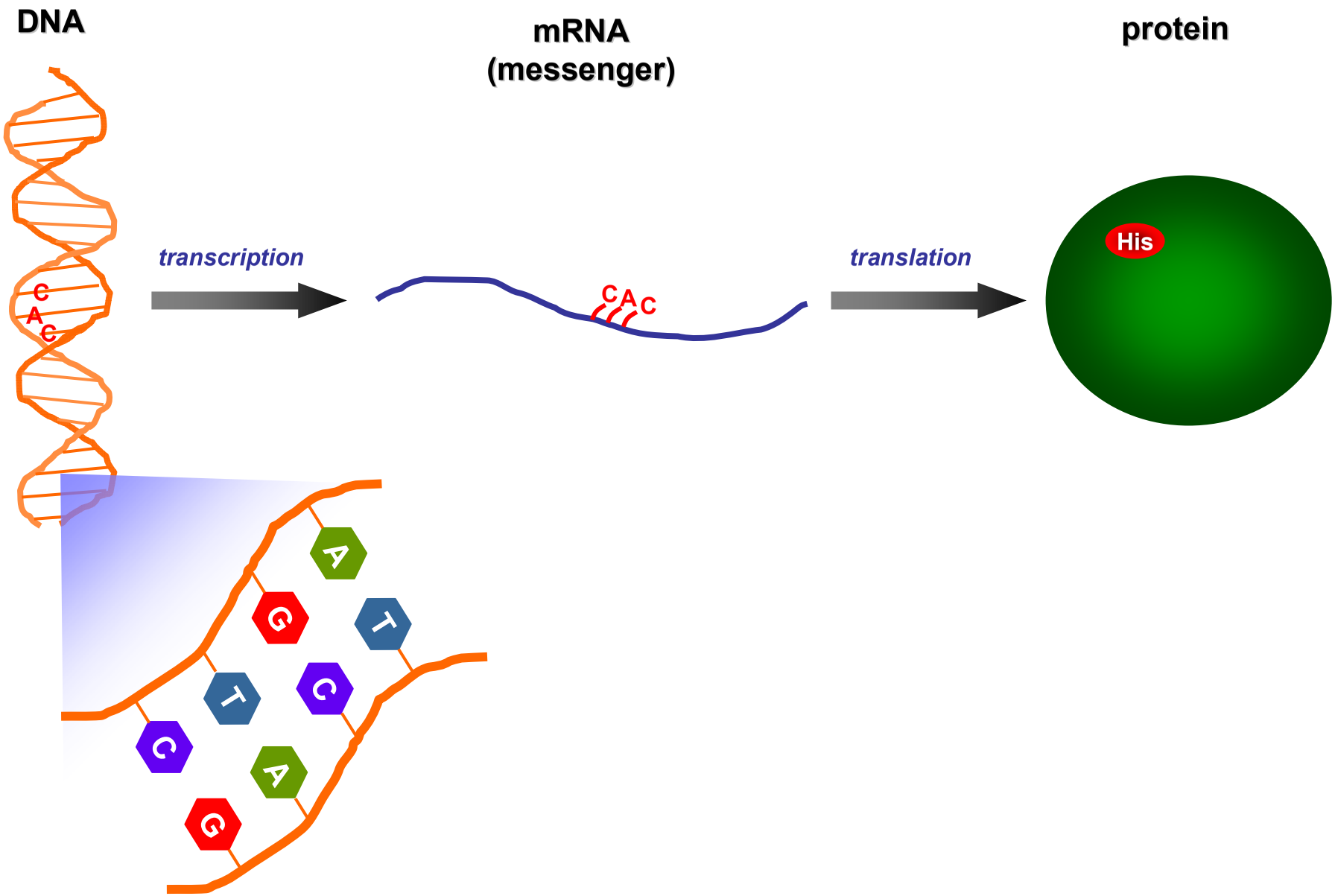
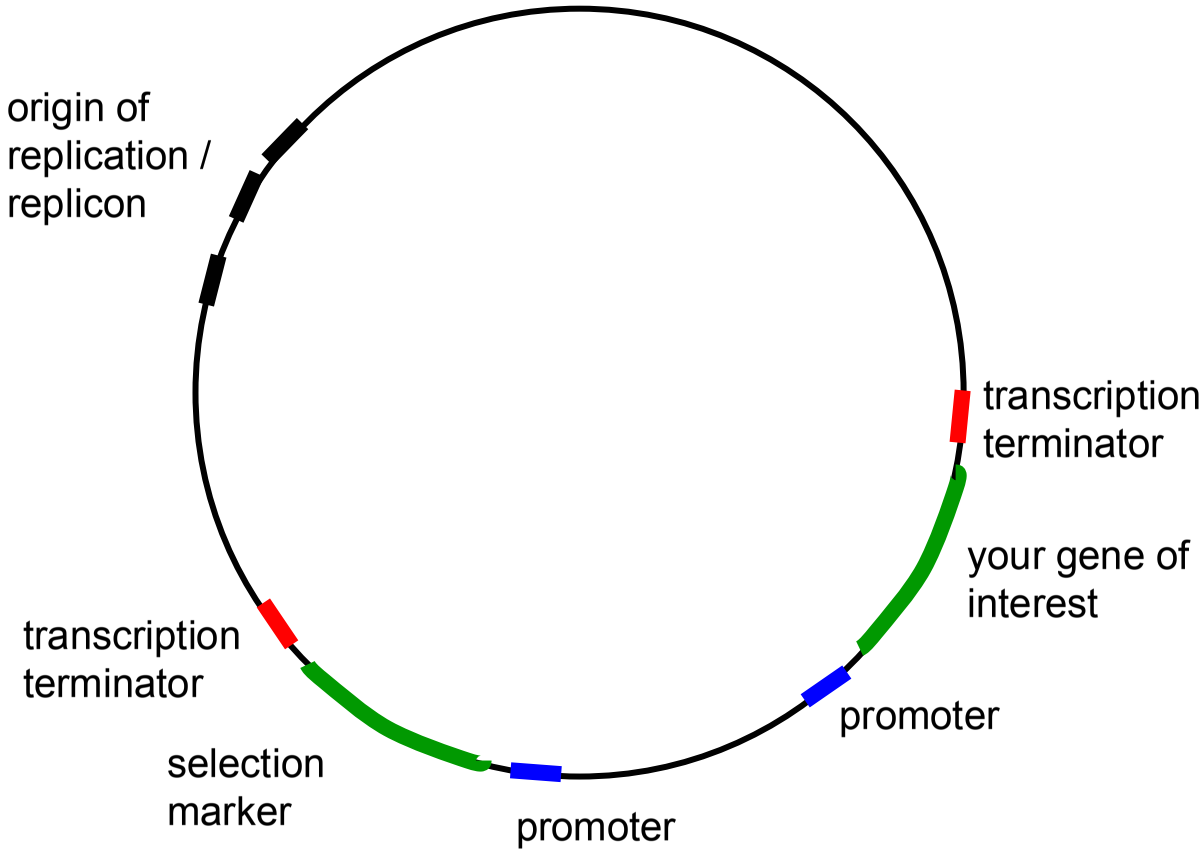
- Design
- Construction
- Characterization

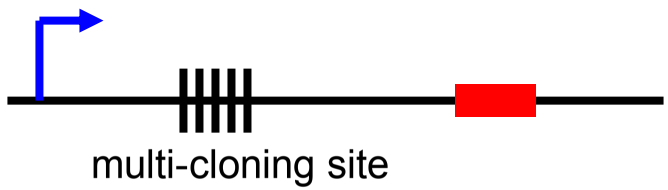

DNA
mRNA
(messenger)
protein
transcription
translation
C
A
C
C
A
C
His
A
G
T
T
C
C
A
G

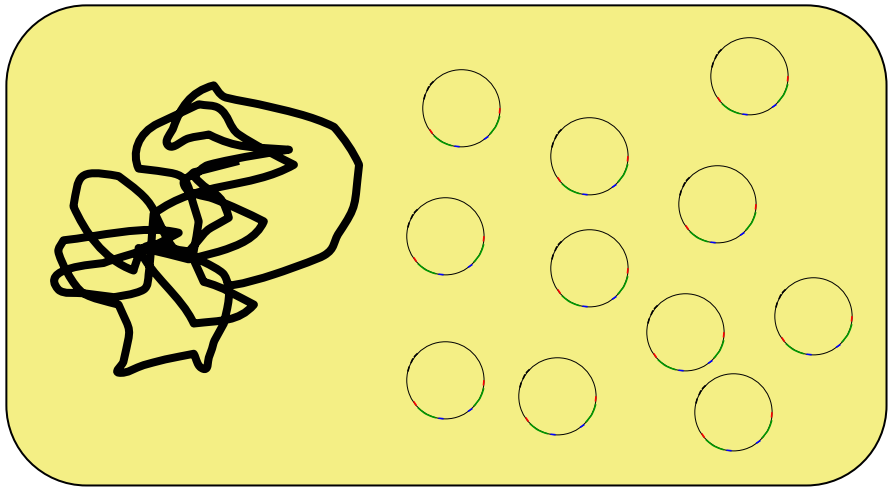
Plasmids

- replicon
- copy number
- incompatibility
- selection marker



Expression Plasmid



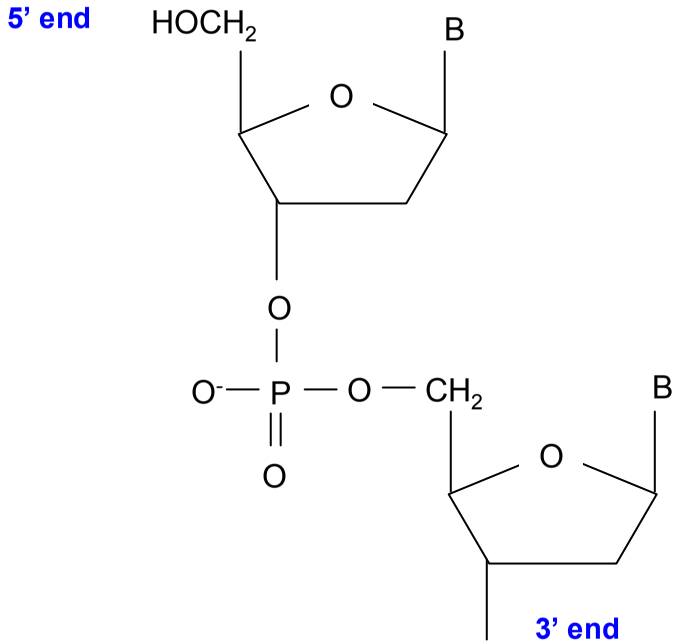


Plasmids

vs.

Chromosomal Insertions

De Novo DNA Synthesis



DNA has directionality:
5' GATC 3'
is different than
3' GATC 5'

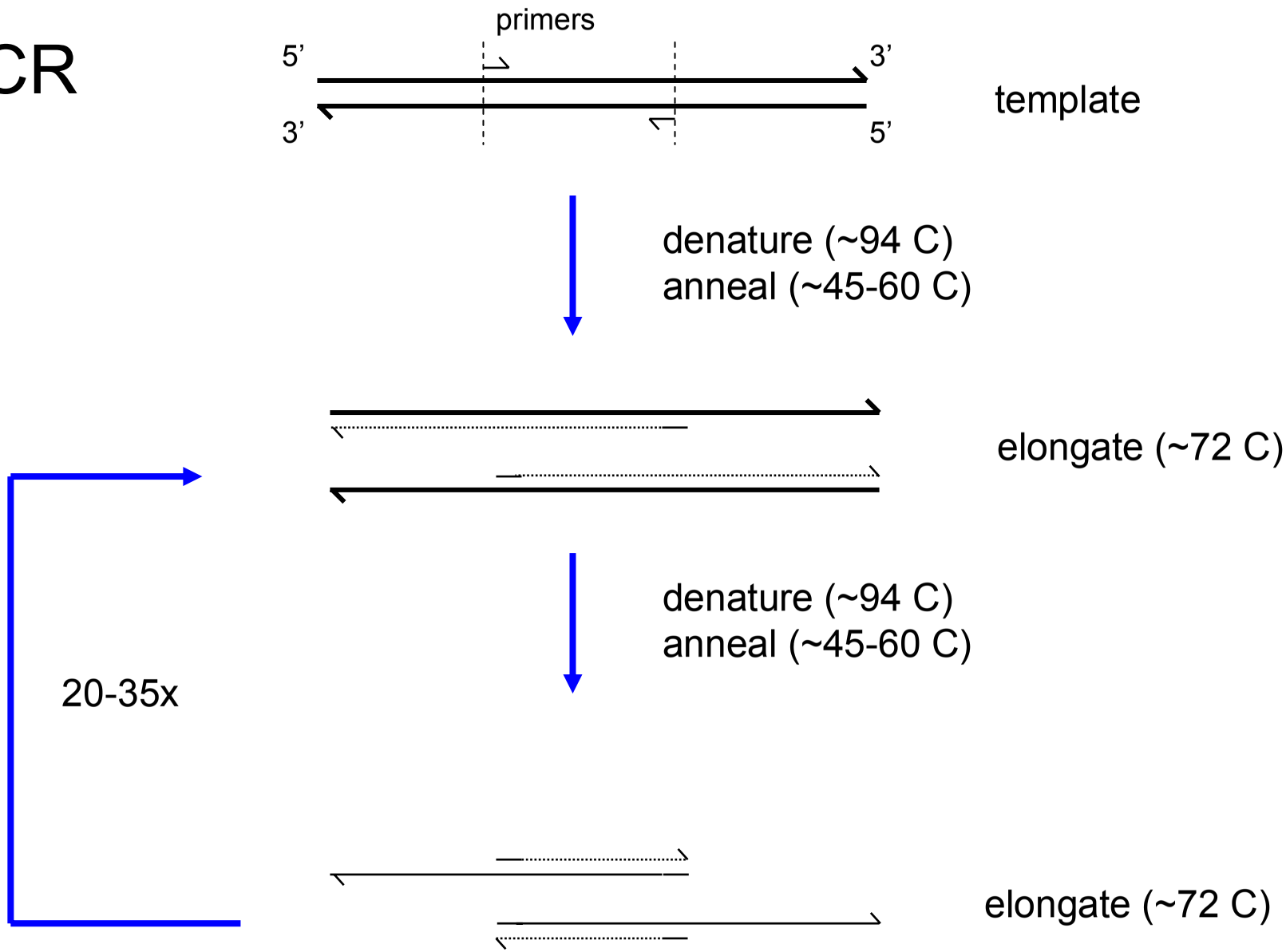
DNA is chemically synthesized 3' to 5'

DNA is biologically synthesized 5' to 3'

De novo DNA synthesis is currently limited to ~200 nt
Due to error rates in synthesis must purify over ~70 nt

For de novo synthesis over 200 nt, must use advanced strategies such as assembly

PCR

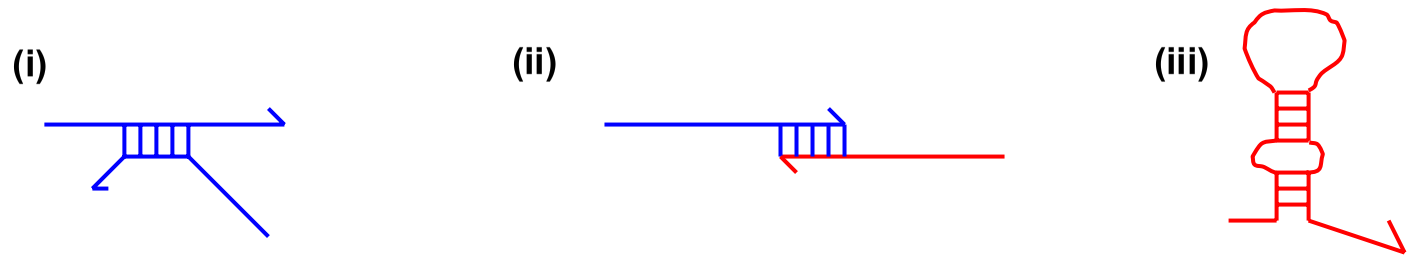


PCR requires: template, primers, thermostable polymerase

polymerases vary with fidelity, editing activity, speed

Primer Design

- Primers should have Tms between 45-65 C
- Primer sets should have similar Tms
- Primer sequences flank the region to be amplified, this may limit primer sequence space
- Primer sequences should be designed to minimize (i) homodimerization, (ii) heterodimerization, (iii) hairpin formation

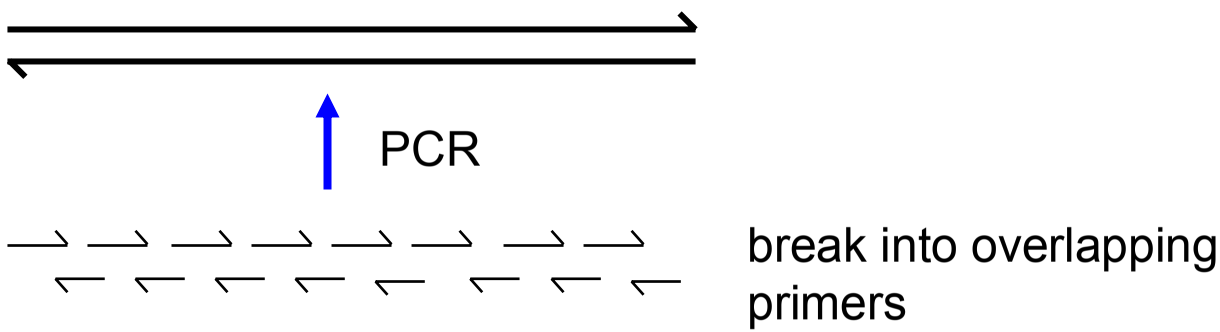


- Primer design should take into account non-specific binding to other regions on the DNA template (longer is not always better)

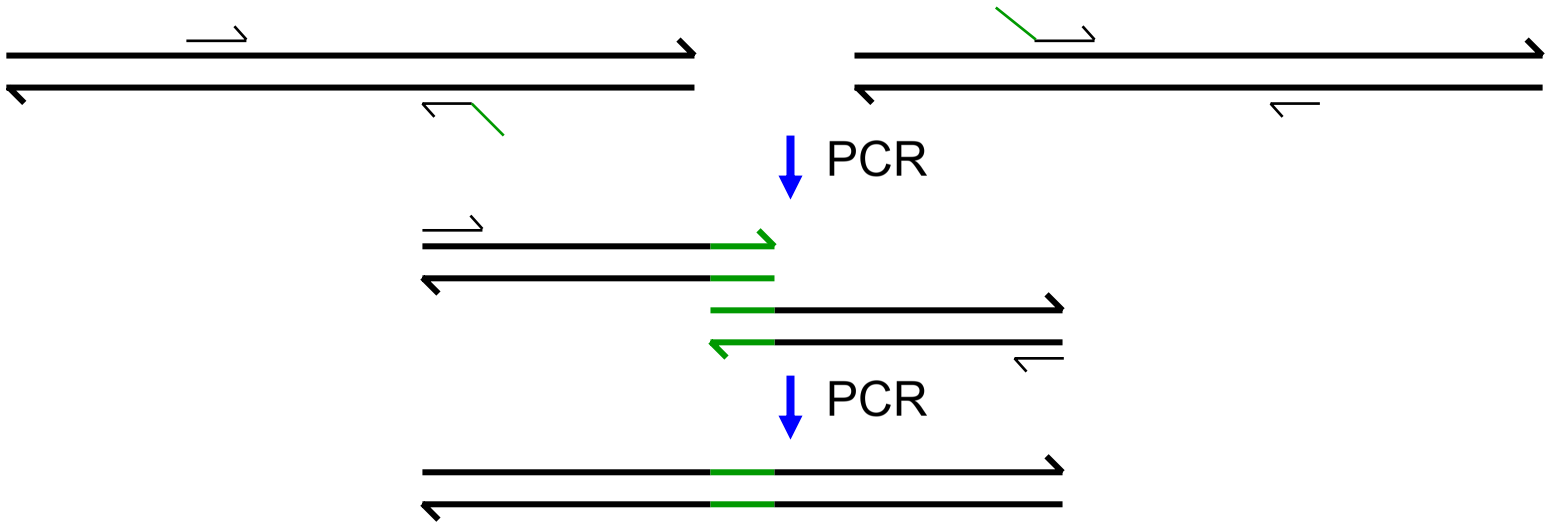
Modifications to PCR

1. Addition of new sequences within primers

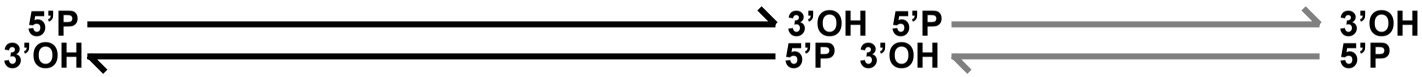
2. PCR assembly



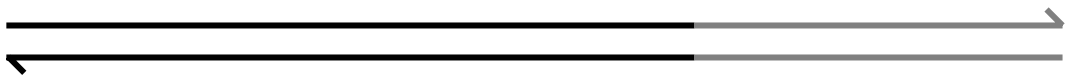
3. Overlap extension



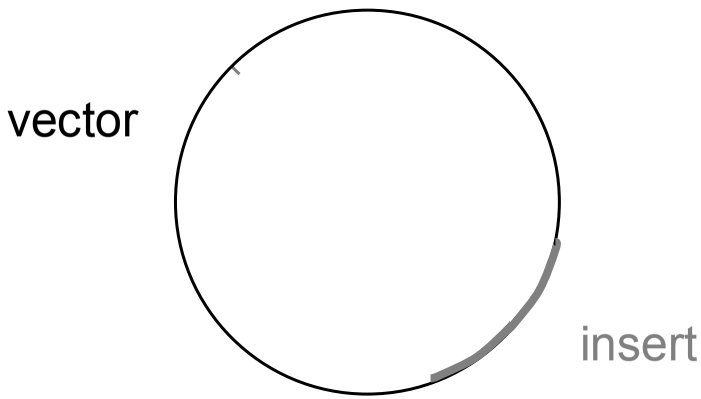
Ligation reactions



Intermolecular reaction
(effective at high concentrations)



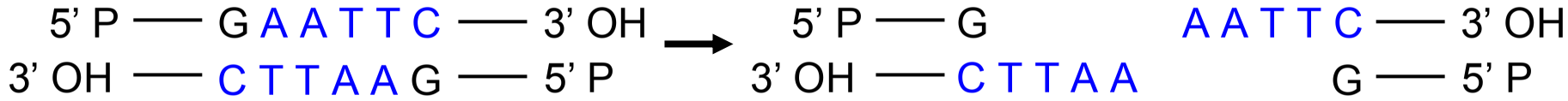
Intramolecular reaction
(effective at lower concentrations)



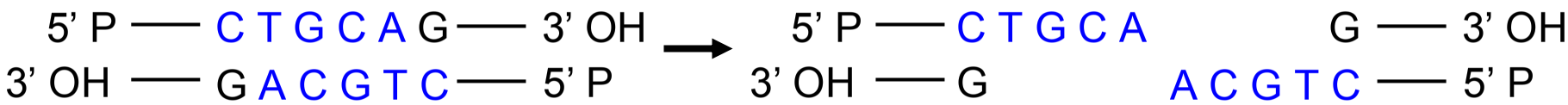
1 of many possible products – linear, circular, homo and heterodimers

Cloning

5’ protruding ends (EcoRI)

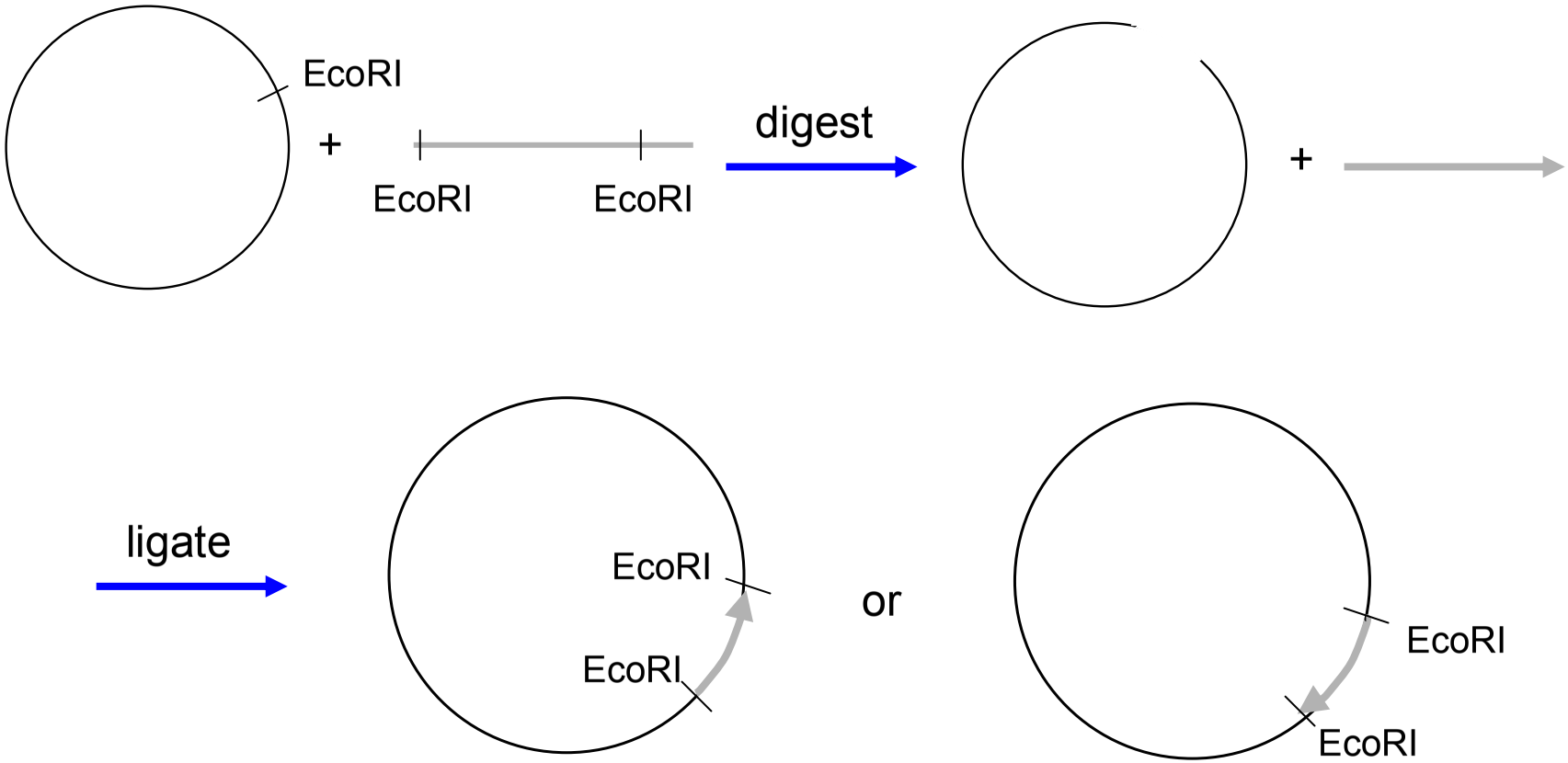


3’ protruding ends (PstI)



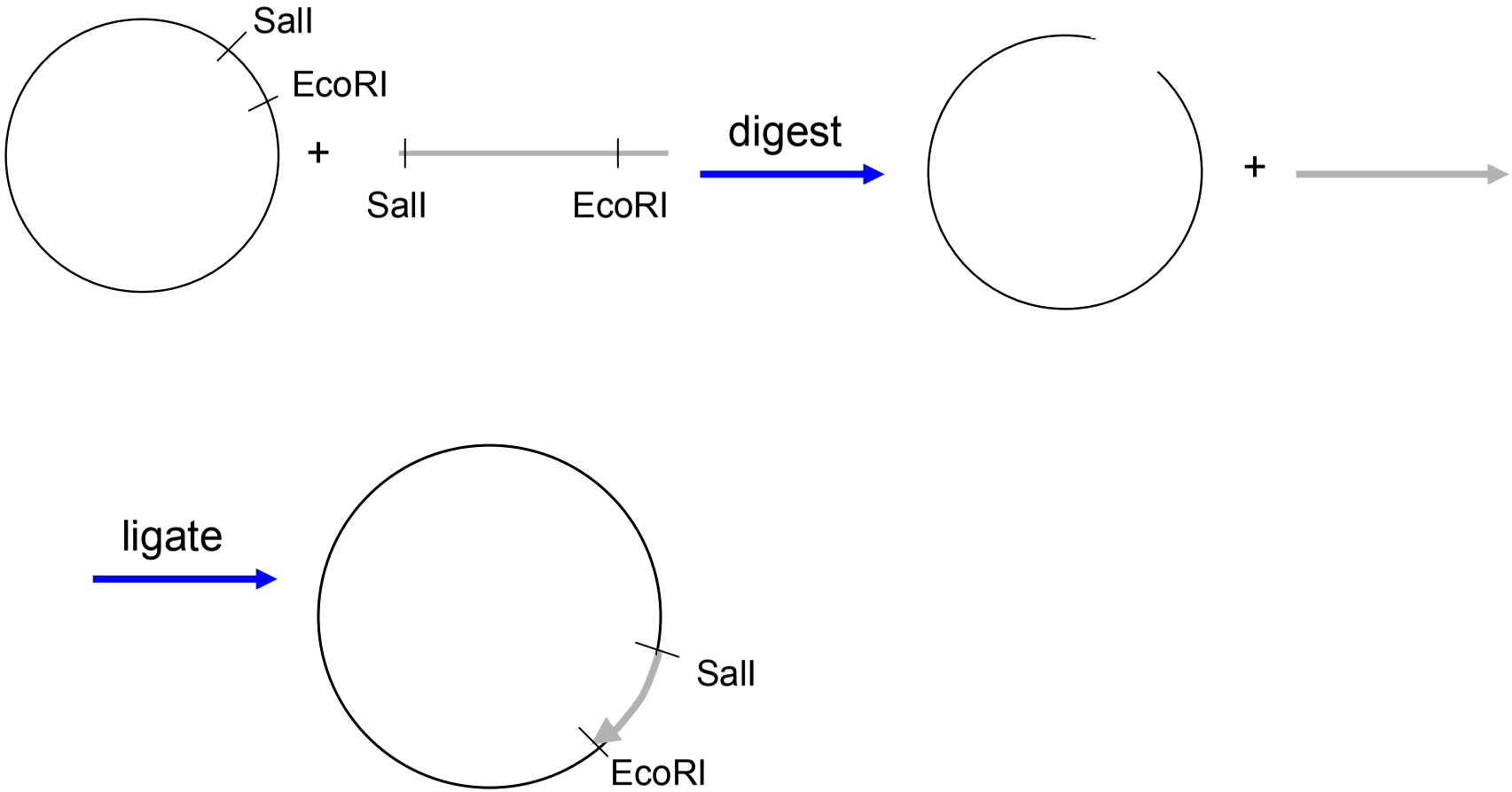
Cloning

Non-directional cloning (single restriction site)



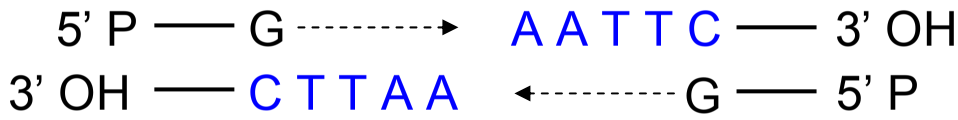
Cloning

Directional cloning (two non-compatible restriction sites)

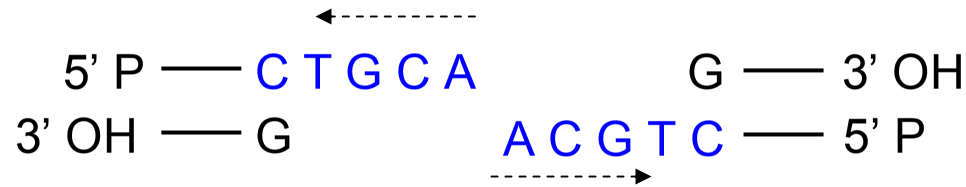


Blunt end cloning…

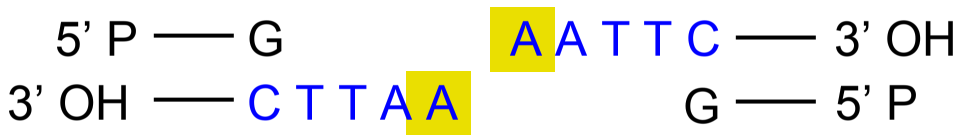
- Klenow fragment – used to fill in 5’ protruding ends



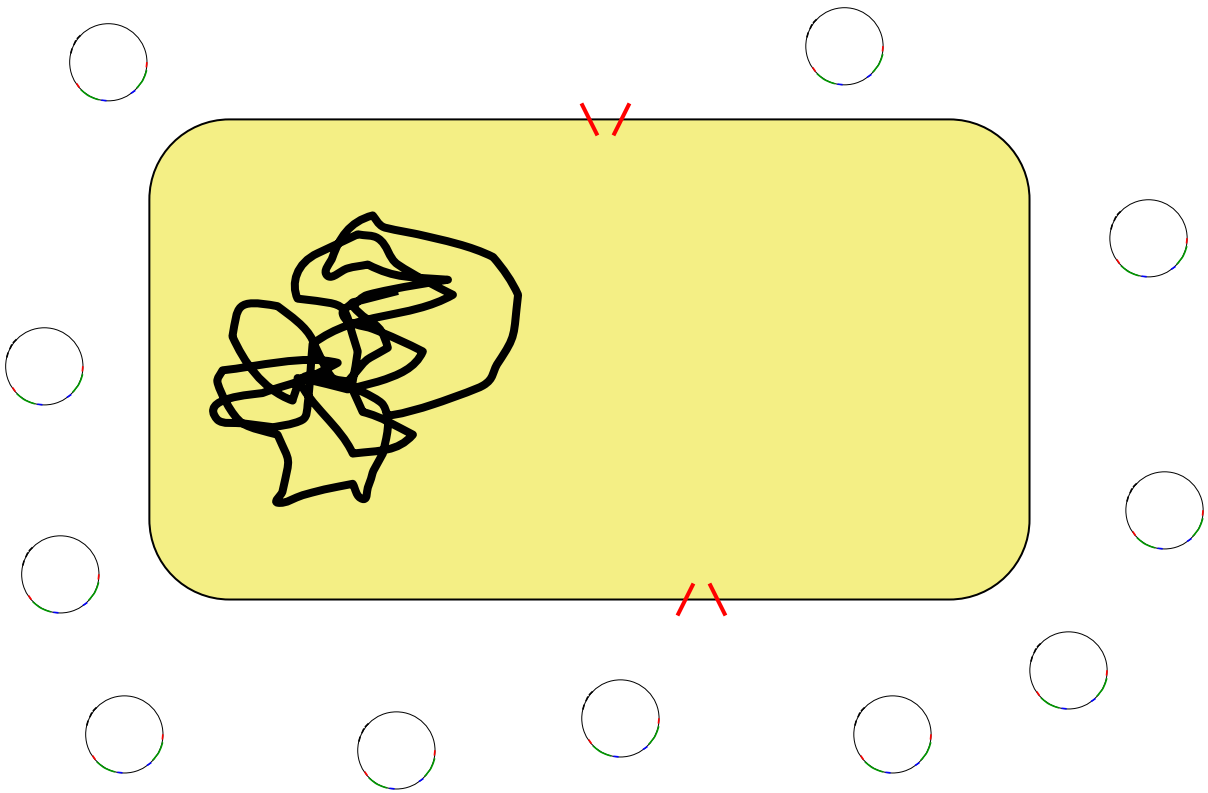
- Exonuclease I – removes single-stranded nts in 3’ -> 5’



- Phosphatase – removes 5’ phosphate on a DNA strand



Transformations



Chemical methods

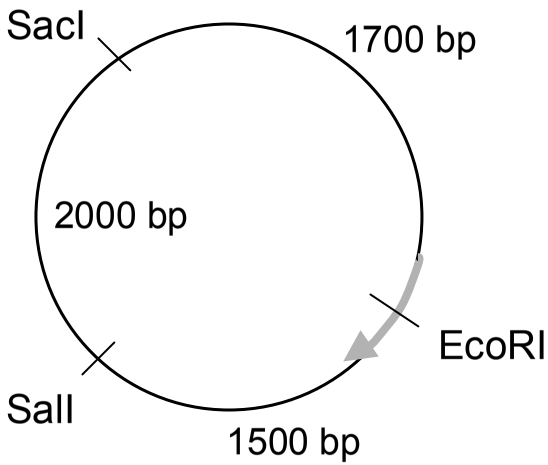
ice cold salt solutions, heat shock; efficiencies ~10^5-10^6 colonies/ug scDNA

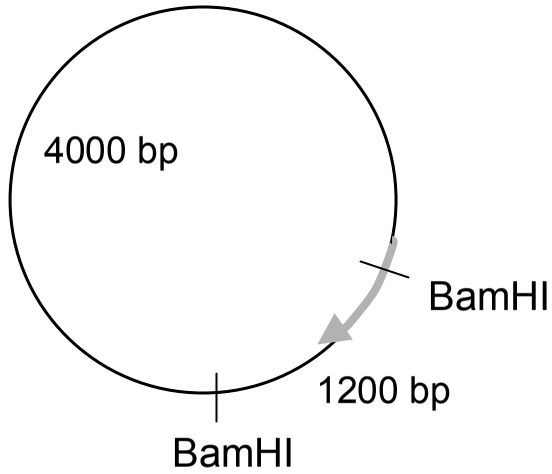
Physical methods

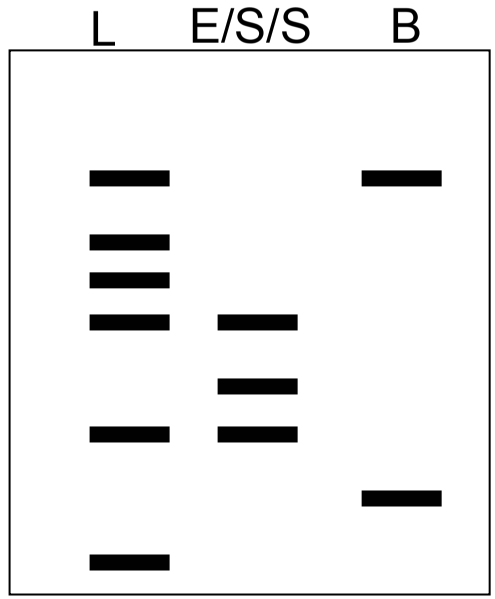
ice cold salt-free solutions, electrical charge; efficiencies ~10^9-10^{10} col/ug scDNA

Screening

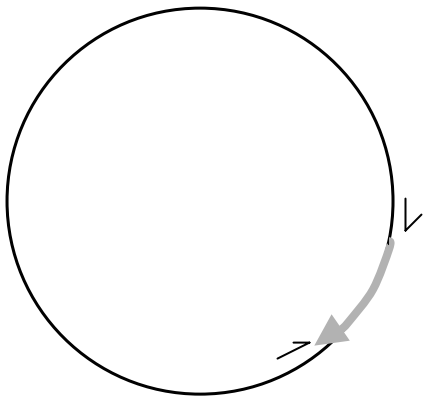
Restriction mapping







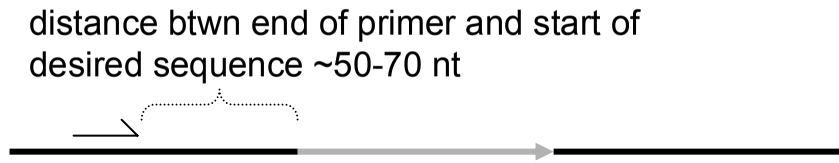
Colony PCR



Confirm change in PCR fragment in cloned construct

Sequencing

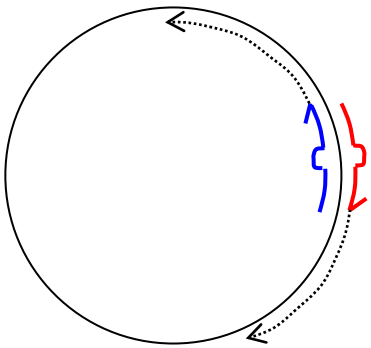
Confirm sequence of cloned fragment



Altering DNA Sequences

Rational, targeted alteration through site-directed mutagenesis

Small changes to a DNA sequence (~1-5 nt)



1. Design complementary primers with altered DNA sequence (can be specific sequence or randomized sequence)
2. Perform PCR on entire plasmid
3. Digest parent plasmid with restriction enzyme that targets methylated DNA
4. Transform into appropriate host

Can perform on fragment and follow with cloning

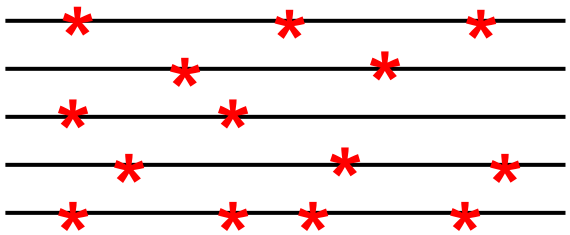
Directed evolution

Random mutagenesis

Perform PCR under conditions that increase the mutation rate of the polymerase

Recombination

family of related sequences

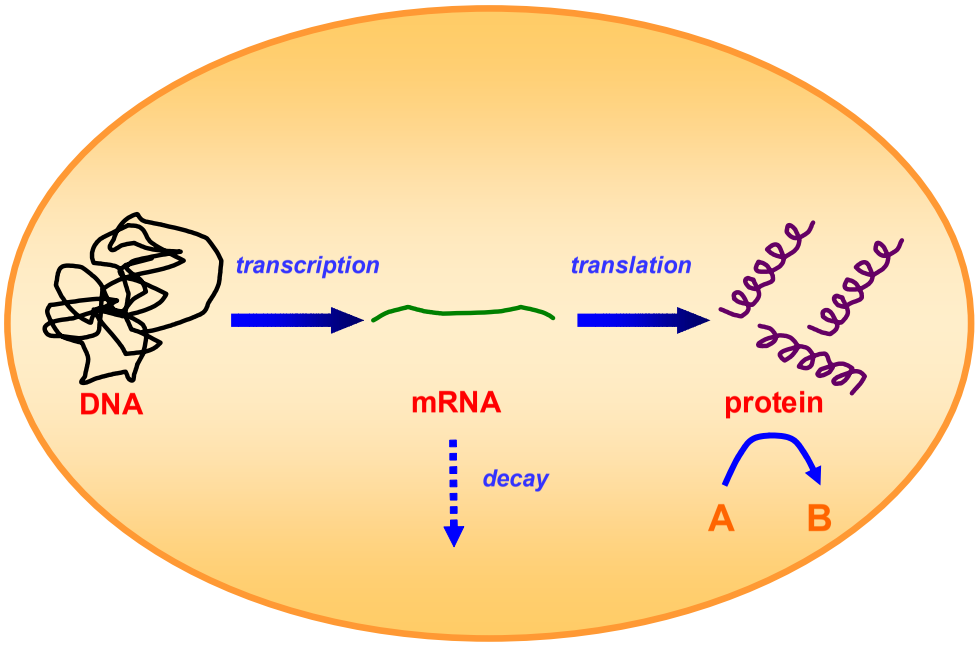


- random digestion of fragments and assembly
- directed crossover events and assembly

Pulling out functional members of large libraries

- *selection*: an assay that links the desired property of the molecule to cell growth
- *screen*: an assay that links the desired property of the molecule to a detectable output signal

Characterization Tools

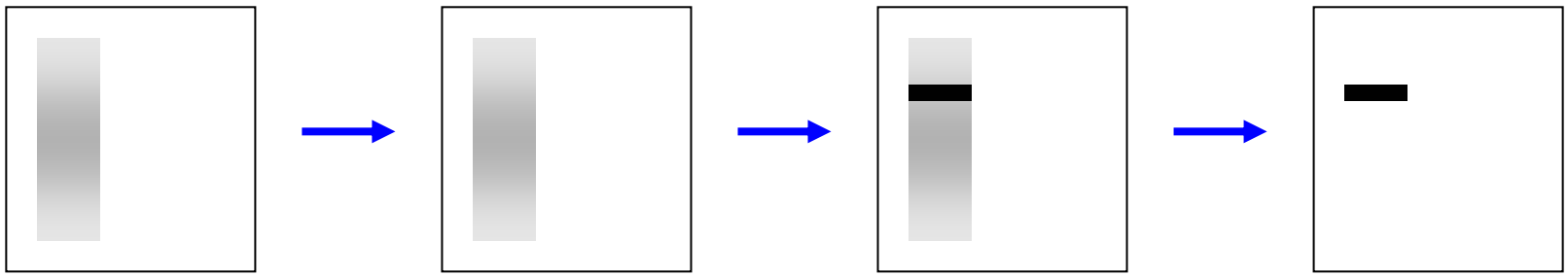


Purification of cellular constituents

- DNA
- RNA
- proteins
- metabolites

mRNA

Northern blot analysis



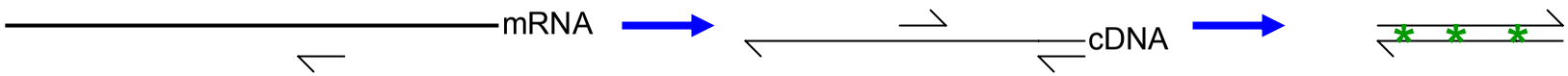
run RNA out on gel

transfer RNA to membrane

hybridize with labeled probe for transcript of interest

detect and quantify the probe signal

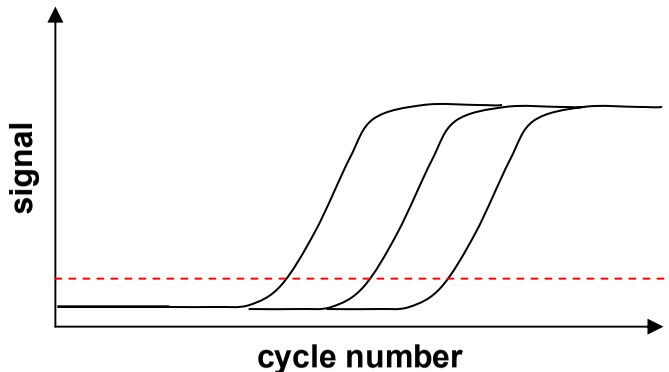
Real-time quantitative PCR (qRT-PCR)



make cDNA copies of the RNA

amplify region of the cDNA

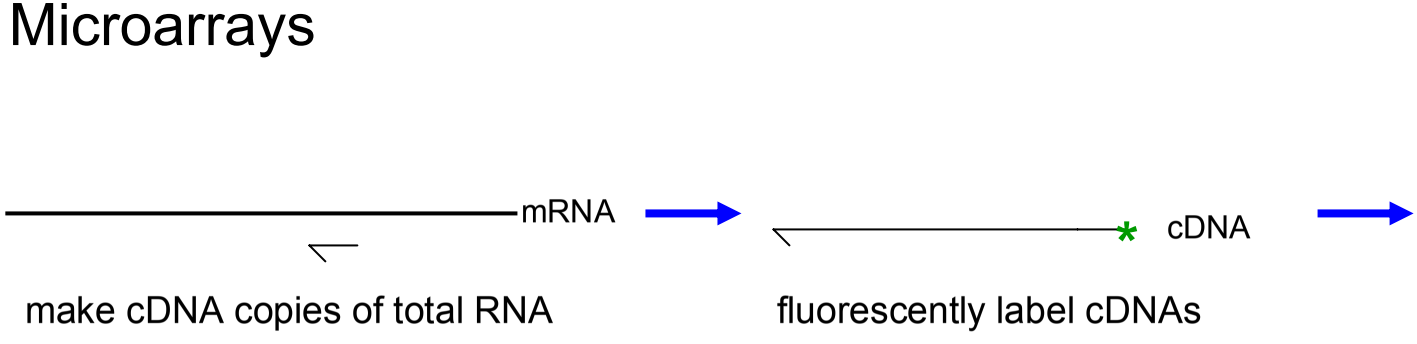
quantify amplification during PCR



Differences in relative starting amounts of cDNA template are reflected in differences in cycle numbers when exponential phase of amplification is observed

mRNA

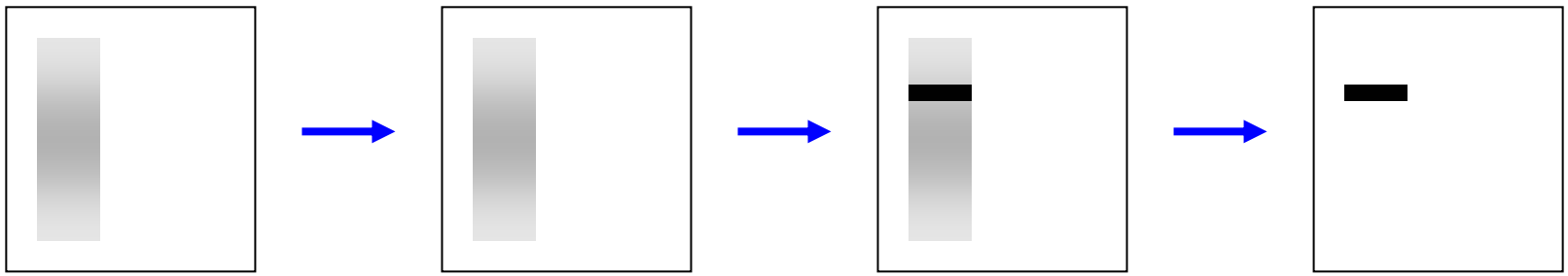
Microarrays



Microarray methods have been adapted to detecting proteins and metabolites

Proteins

Western blot analysis



run protein out on gel

transfer protein to membrane

hybridize with labeled probe to protein of interest (antibody)

detect and quantify the probe with a secondary antibody

Analytical techniques

Commonly used – liquid chromatography-mass spectrometry (LC-MS), tandem MS-MS, 2-dimensional gel electrophoresis (2D-GE)

MS is used for identification

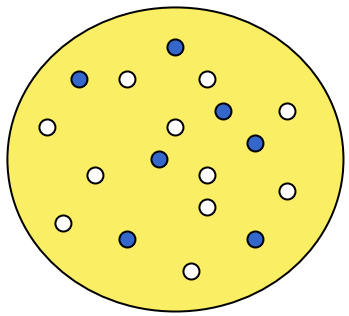
LC, gas chromatography, GE, and capillary electrophoresis are used to separate and purify

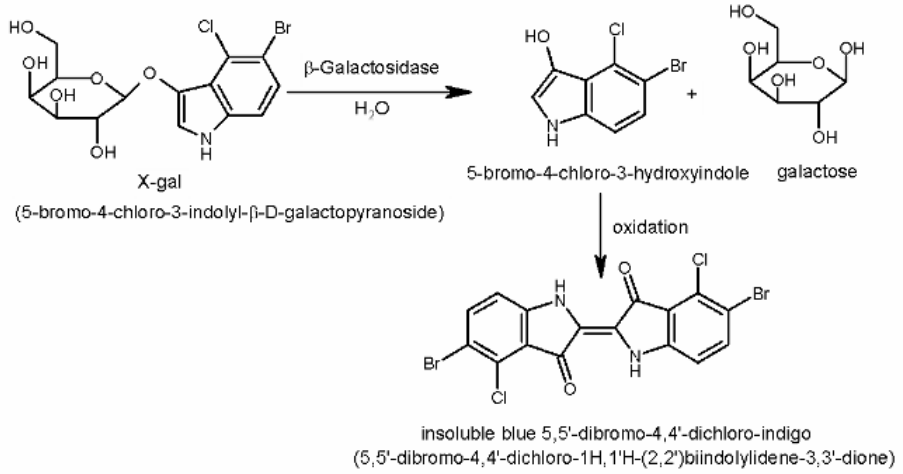
Activity and reporter assays

Reporter enzymes

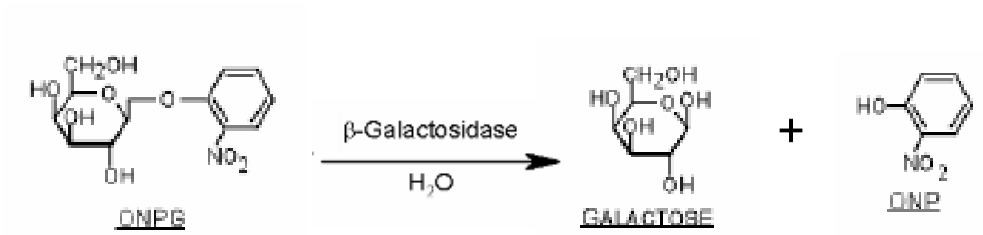
Most commonly used is β-galactosidase (encoded by the *lacZ* gene)

Plate-based assay





Solution / quantitative assay



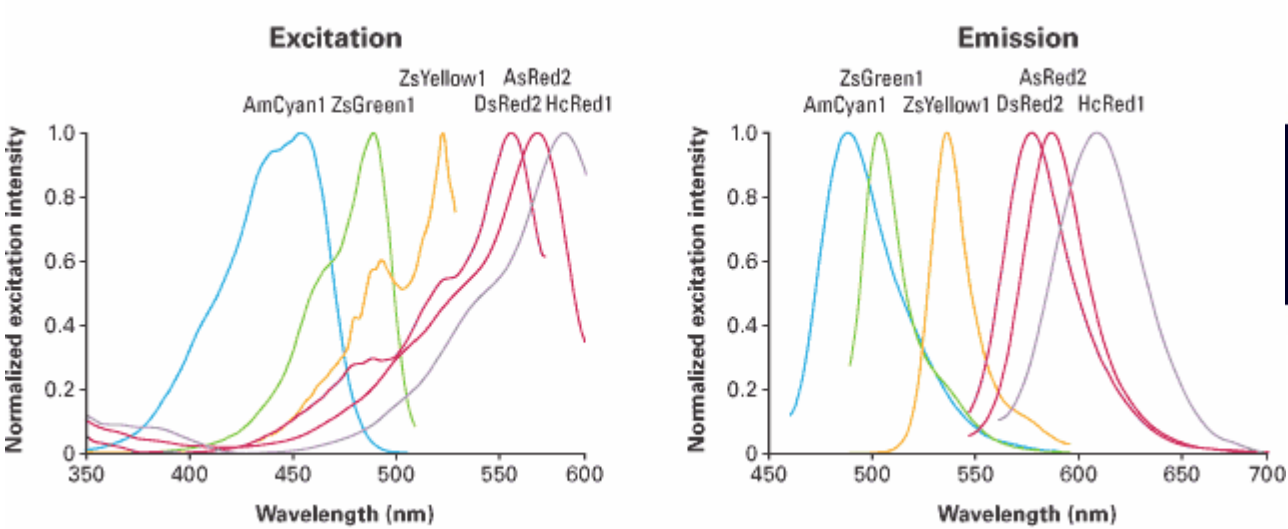
yellow

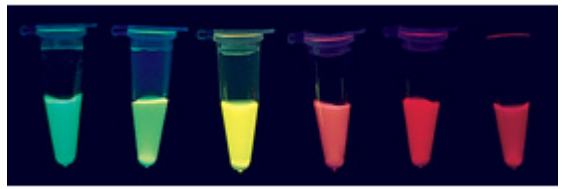
Other commonly used reporter enzymes are luciferases

Activity and reporter assays

Reporter proteins

Fluorescent reporter proteins are commonly used and require no substrate





GFP was original fluorescent reporter protein

Researchers have spent significant effort generating: color-shifted variants, more rapidly folding and degrading variants, monomeric forms, brighter fluorescence, resistant to photobleaching, optimized for different organisms

FRET applications

Activity and reporter assays

Use of fluorescent reporter proteins as readouts of cellular components and networks